

# Complete nucleotide sequence of the low copy number plasmid pRAT11 and replication control by the RepA protein in *Bacillus subtilis*

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**Summary.** The 2.6 kb kanamycin-resistant ( $K_m'$ ) plasmid, pRAT11, was constructed using both the replication determinant (*repA*) region of the 10.8 kb tetracycline-resistant ( $T_c'$ ) low copy number plasmid pTB52 and another fragment (0.9 kb) that contained solely the  $K_m'$  gene of pUB110. The complete nucleotide sequence of this plasmid was determined. The *repA* region contained a large open reading frame encoding RepA protein (396 amino acid residues). *In vitro* transcription and translation of the *repA* gene were confirmed. RepA protein was shown to be indispensable for plasmid replication, and acted in *trans* on DNA. The part of the *repA* gene encoding the specific recognition region of the RepA protein was located and contained 3.5 direct repeats of 24 bp (GGTTTCAAAAT-GAAACGGTGGAG). Upstream and downstream of the direct repeats were the recognition sequence (TTATC-CACA) of the *Escherichia coli* DnaA protein and an AT-rich region, respectively. The replication control mechanism of the low copy number *Bacillus* plasmid is discussed.

**Key words:** Nucleotide sequence of pRAT11 - Low copy number plasmid - *Bacillus subtilis* - RepA protein - Direct repeat

## Introduction

A 26.5 kb drug resistance plasmid, pTB19, resistant to kanamycin ( $K_m'$ ) and tetracycline ( $T_c'$ ), has been isolated from a thermophilic *bacillus* (Imanaka et al. 1981a), and found to contain two different replication determinants, *repA* and *repB* (Imanaka et al. 1984). One replication determinant, *repB*, was functional in both *Bacillus subtilis* and *Bacillus stearothermophilus*. The nucleotide sequence of the *repB* region has been determined (Muller et al. 1986), and the replication control mechanism of the *repB* plasmid (high copy number) has been studied (Ano et al. 1986). The other replication determinant, *repA*, functions only in *B. subtilis*, and the *repA* plasmid has a low copy number (about eight copies per chromosome; Imanaka et al. 1984).

Most of the plasmids that are widely used for *B. subtilis* are high in copy number, whereas low copy number plasmids are scarce. Although the replication origin (*ori*) region of the *B. subtilis* chromosome has been analysed (Seiki et al. 1979), an *ori* plasmid has not been successfully constructed because of its inhibitory effect on the host cell (Seiki et al.

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1981). Therefore, the *repA* plasmid would be a useful model system for investigating the replication control of low copy number plasmids in *B. subtilis*.

This paper gives the nucleotide sequence of the *repA* region and demonstrates that the RepA protein, acting in *trans*, is required for plasmid replication.

## Materials and methods

**Bacteria and plasmids.** The bacterial strains and plasmids used are listed in Table 1. pPF201 and pPF301 contain the penicillinase gene, *penP*, lacking a promoter and were used as promoter-probe vectors. Plasmid pMC1871 was used as a source of the *lacZ* gene.

**Preparation of plasmid DNA.** Plasmid DNA was prepared either by the alkaline phenol procedure or the cleared lysate method, followed by CsCl-ethidium bromide equilibrium density gradient centrifugation as described previously (Mutsumura et al. 1984; Imanaka et al. 1985).

**Transformation.** *B. subtilis* was transformed with plasmid DNA by the competent cell or protoplast procedures as described (Imanaka et al. 1981a; Aiba et al. 1983).

**Cleavage of DNA with restriction enzymes, repair of cohesive ends and ligation of DNA.** Treatment of DNA with restriction enzymes (*Aat*I, *Acc*I, *Bgl*II, *Bsr*II, *Bst*NI, *Eco*RI, *Hind*III, *Hinf*I, *Pst*I, *Rsa*I, *Sma*I, *Sty*I, *Taq*I, etc.), repair of cohesive ends with the large fragment of DNA polymerase I and ligation of DNA with T4 DNA ligase were done following the protocols of the manufacturers. The enzymes used in these experiments were purchased from commercial suppliers.

**Gel electrophoresis for DNA isolation.** Agarose gel electrophoresis and DNA isolation from low melting point agarose gels are described elsewhere (Imanaka et al. 1985).

**Detection of penicillinase-positive colonies on plates and penicillinase assay.** Penicillinase was assayed by the iodometric method as described previously (Imanaka et al. 1981b). The detection of penicillinase-positive colonies on plates has also been described earlier (Imanaka et al. 1981b).

**Detection of  $\beta$ -galactosidase-positive colonies on plates.**  $\beta$ -Galactosidase-positive colonies were detected as blue colo-

Table 1. Bacterial strains and plasmids

Strain	Characteristics		Reference
<i>Bacillus subtilis</i> M1113	<i>arg</i> -15 <i>trpC2</i> <i>r<sub>M</sub></i> <i>m<sub>M</sub></i>		Imanaka et al. (1981a)
<i>Bacillus subtilis</i> M1112	<i>leuA8</i> <i>arg</i> -15 <i>thr-5</i> <i>recE4</i> <i>r<sub>M</sub></i> <i>m<sub>M</sub></i>		Imanaka et al. (1981b)
Plasmid	Molecular size (kb)	Characteristics*	Reference
pTB19	26.5	<i>Km'</i> <i>Tc'</i> <i>repA</i> <i>repB</i> , low copy number	Imanaka et al. (1981a)
pTB52	10.8	<i>Tc'</i> <i>repA</i> , low copy number	Imanaka et al. (1984)
pRA1	7.4	<i>Km'</i> <i>repA</i> , low copy number	This work
pRAT1	3.5	<i>Km'</i> <i>repA</i> , low copy number	This work
pRAT11	2.6	<i>Km'</i> <i>repA</i> , low copy number	This work
pTB902	4.2	<i>Tc'</i> <i>repB</i> , high copy number	Imanaka et al. (1984)
pTB20	4.3	<i>Tc'</i>	Imanaka et al. (1981a)
pPF201	7.7	<i>Km'</i> , promoterless penicillinase gene	Imanaka et al. (1986)
pPF201-PA	8.1	<i>Km'</i> , pPF201 + <i>Hinf</i> I fragment (-228-218)	This work
pPF301	6.0	<i>Cm'</i> , promoterless penicillinase gene	Ano et al. (1986)
pPF301-PA	6.5	<i>Cm'</i> , pPF301 + <i>Hinf</i> I fragment (-228-218)	This work
pMC1871	7.8	<i>Tc'</i> , <i>lacZ</i> (active C-terminal portion)	Shapiro et al. (1983)
pC194	2.9	<i>Cm'</i> , constructed from pHV14	Imanaka et al. (1982)
pMCK82	8.8	<i>Km'</i> <i>Tc'</i> , pMC1871 + <i>Km'</i> gene	This work
pHK96	11.7	<i>Cm'</i> <i>Km'</i> , pMCK82 + pC194	This work
pHKZ16	12.5	<i>Cm'</i> <i>Km'</i> , <i>repA</i> - <i>lacZ</i> fusion, pHK96 + <i>Rsa</i> I fragment of pRAT11	This work
pATB61	6.8	<i>Km'</i> <i>Tc'</i> <i>repB</i> , pRAT11 + pTB902	This work
pATB61D	4.8	<i>Km'</i> <i>repA</i> <i>repB</i>	This work
pATB61D <sub>Aco</sub>	4.1	<i>Km'</i> <i>repA</i> <i>repB</i>	This work
pATB61dTaq	3.9	<i>Km'</i> <i>repA</i> <i>repB</i>	This work
pATB61dSly-Bst	4.5	<i>Km'</i> <i>repA</i> <i>repB</i>	This work
pBR322kan	3.5	<i>Km'</i> gene of pUB110 + pBR322	Matsumura and Aiba (1985)

\* Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; r, resistance; repA and repB, different replication determinants from pTB19

ries on L-agar containing 40 µg/ml of Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (Miller 1972).

**DNA sequencing.** DNA sequencing was performed by the dideoxy method (Messing 1983), using an M13 sequencing kit (Takara Shuzo Co., Kyoto, Japan).

**Assessment of plasmid copy number.** The plasmid copy number was assessed as described by Muller et al. (1986).

## Results

### Construction of small repA plasmids

To analyse the structure and function of the repA region, deletion plasmids were constructed from pTB52. Since a 6.5 kb *Eco*RI fragment of pTB52 contained the repA region (Imanaka et al. 1984), this fragment was ligated with a 930 bp *Eco*RI fragment that contained only the *Km'* gene of pBR322kan (Matsumura and Aiba 1985). The nucleotide sequence of *kan* has been determined by our group (Matsumura et al. 1984). The small plasmid obtained in *B. subtilis* M1113 was designated as pRA1 (7.4 kb, *Km'*; Fig. 1). Using *Tag*I and T4 DNA ligase, the deletion plasmid pRAT1 (3.5 kb, *Km'*) was constructed (Fig. 1). The 2.6 kb *Eco*RI fragment containing the repA region of pRAT1 was digested from both ends by exonuclease *Bal*31, and was joined by blunt end ligation with the *Km'* fragment whose *Eco*RI sites had been repaired by DNA polymerase I (large frag-

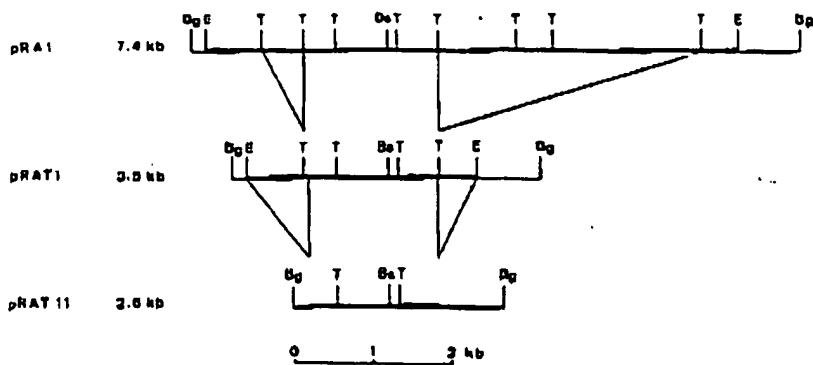
ment). The ligation mixture was used to transform *B. subtilis* M1113. The smallest plasmid thus obtained was designated as pRAT11 (2.6 kb, *Km'*; Fig. 1). The copy number of pRAT1 was as low as that (about eight copies per chromosome) of the original plasmid pTB52.

### Nucleotide sequence of the repA region

The complete nucleotide sequence of the repA region (1,631 bp) was determined (Fig. 2). The A+T content of this region was fairly high (68 mol%). There was only one large open reading frame (nucleotides 1-1,188), starting from a GTG codon, which can encode a protein of 100 amino acids or more in three reading frames of each strand. Nine bases upstream from the initiation codon, there was a possible Shine-Dalgarno (SD) sequence (GGAGG, -13-9) which exhibits complementarity with the 3'-end of *B. subtilis* 16 S rRNA (HO-UCUUUCCUCCACUAG; McLaughlin et al. 1981; Moran et al. 1982). Consequently, a coding sequence of 1,188 nucleotides, encoding 396 amino acids (molecular weight 47,493) could be expected. About 3.5 direct repeats (547-634) of 24 bp (GCTTTCAAAAT-GAAACGGTGGAG) were found in the open reading frame (Fig. 2).

### Detection of promoter activity in vivo

Since potential promoters which resemble the consensus sequence (TTGACA for the -35 region and TATAAT for



**Fig. 1.** Restriction endonuclease cleavage maps of pRA1 and its deletion plasmids. **Bold** and **light** lines represent the fragment containing the *repA* region from pTBS2, and the 930 bp fragment that contains the *Km'* gene of pBR322kan, respectively. Bg, *Bgl*II; *Bpu*II; E, *Eco*RI; T, *Tag*I.

Fig. 2. Nucleotide sequence of the *repA* region of pRAT11. The position of the 1st nucleotide of the coding region is defined as +1. The amino acid sequence of the coding region is given below. The Shine-Dalgarno (SD) sequence and a putative promoter sequence (-35 and -10 regions) are shown. Regions of direct and inverted repeats are indicated by solid arrows (→→) and dotted arrows (.....,.....), respectively. The binding sequence of the DnaA protein is boxed. Asterisks indicate a stop codon.

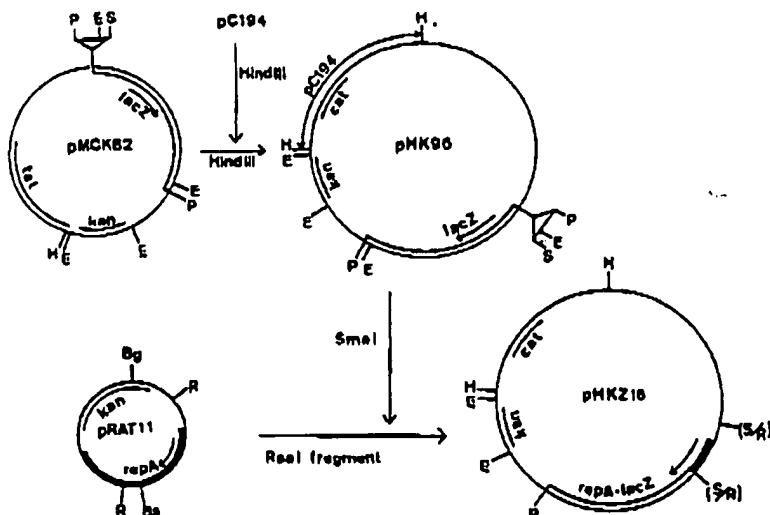


Fig. 3. The scheme for gene fusion between *repA* and *lacZ*. Arrows inside the circles show the direction of translation. The bold line represents the *repA* gene. Bg, *Bgl*II; Bs, *Bst*III; E, *Eco*RI; H, *Hind*III; P, *Pst*I; R, *Rsa*I; S, *Sma*I. (S/R) means disappearance of restriction sites due to blunt end ligation

the -10 region for *B. subtilis*; Moran et al. 1982) were not found in front of the open reading frame, promoter activity in vivo was examined using the promoter-probe vector plasmid pPF201. The *Hinf*I fragment (-228-218) containing the initiation codon and the upstream region was repaired with DNA polymerase I (large fragment) and ligated with a linearized pPF201 fragment which had been digested with *Bam*HI and repaired. The recombinant plasmid thus obtained was designated as pPF201-PA. Strain MI113 of *B. subtilis* carrying pPF201-PA exhibited a large halo in the penicillinase plate test, although the pPF201 carrier did not. This shows that the *Hinf*I fragment contains an active promoter. A putative promoter sequence is TTTACA (-60-55) for the -35 region and ATTTCT (-36-31) for the -10 region, and the spacer is 18 bp.

#### Gene fusion between the open reading frame and the *lacZ* gene

To examine the in vivo transcription and translation of the open reading frame, a fused gene containing the open reading frame and the *Escherichia coli* *lacZ* gene was constructed (Fig. 3).

Plasmid pMC1871 contains *lacZ* gene sequences encoding an enzymatically active carboxy-terminal portion of  $\beta$ -galactosidase. By inserting the 930 bp *Km'* gene in pMC1871, a new plasmid pMCK82 was constructed, followed by ligation with pC194. The recombinant plasmid, pHK96, is a shuttle vector between *E. coli* and *B. subtilis*. To match the open reading frame and the frame of the *lacZ* gene, the *Rsa*I fragment (about 800 bp) of pRAT11 was subcloned in the *Sma*I site of pHK96. *B. subtilis* MI113 carrying plasmid pHK216 showed blue colonies on Xgal plates, but not when carrying the pHK96 carrier (not shown). This indicates that the open reading frame was transcribed and translated in *B. subtilis*; it was therefore named the *repA* gene.

#### Involvement of the RepA protein in plasmid replication control

A frameshift mutation was introduced into the *repA* gene as shown in Fig. 4 to examine whether or not the RepA protein is involved in plasmid replication control. pTB902

(*Tc'*, *repB* plasmid) was digested with *Eco*RI, repaired with DNA polymerase I (large fragment), and ligated with the *Aat*I fragment of pRAT11. Since the ligation sites of *Aat*I (nucleotide 790) and *Eco*RI, the latter of which was repaired by polymerization, gave rise to two *Eco*RI sites as shown in the middle of the diagram, the recombinant plasmid pATB61 was digested with *Eco*RI and treated with T4 DNA ligase. The ligation mixture was used to transform *B. subtilis* MI113 protoplasts. Three types of transformants were expected, i.e. *Km'* *Tc'* for pATB61, *Km'* *Tc'* for pTB902 and *Km'* *Tc'* for the plasmid with the 4 bp (AATT) insertion shown in the right-hand side of Fig. 4. Many *Km'* *Tc'* and *Km'* *Tc'* transformants were obtained but *Km'* *Tc'* transformants were not obtained at all. In other words, when *repA* was mutated by frameshift, the *repA* replication determinant turned out to be non-functioning. Accordingly, the RepA protein must have been involved in plasmid replication.

To confirm this point we attempted to obtain plasmids with a temperature-sensitive (ts) replication mutation. *B. subtilis* carrying pRAT11 was treated with N-methyl-N'-nitro-N-nitrosoguanidine (35  $\mu$ g/ml), and the plasmid DNA was extracted and used to transform *B. subtilis* MI113. *Km'* transformants were selected on L-agar plus *Kin* (4  $\mu$ g/ml) at 32°C. The transformants were replica plated on L-agar containing *Km* and incubated at 48°C. Out of 500 colonies, 8 transformants could not grow at 48°C. The latter were grown at 48°C on L-agar in the absence of *Km*, and then transferred onto L-agar plus *Km* and incubated at 32°C. None of the strains could grow in the presence of *Km*, even at 32°C. When the plasmid carriers were cultivated in L-broth at 48°C in the absence of *Km*, the plasmid was lost more rapidly from the host cells than was the wild type (pRAT11) (data not shown). Consequently, the plasmids in these transformants were considered to be ts for replication and not ts mutants of the *Km'* gene. Hence, we concluded that the RepA protein is involved in plasmid replication.

#### Cis-trans complementation test for the RepA protein

A cis-trans test for RepA protein was done (Fig. 5). Since the *repA* gene, being cleaved at the *Aat*I site, is split in pATB61, the plasmid can replicate by using the *repB* but

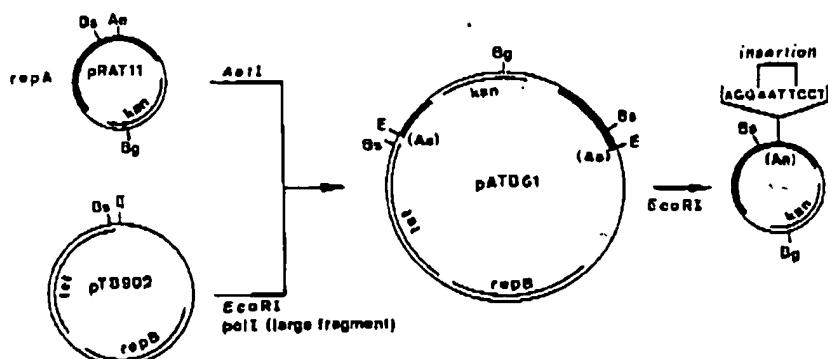


Fig. 4. The scheme for frameshift mutation in the *repA* gene. Thick bars represent the *repA* genes. *Aai*, *AatII*. Other symbols are as in Fig. 3

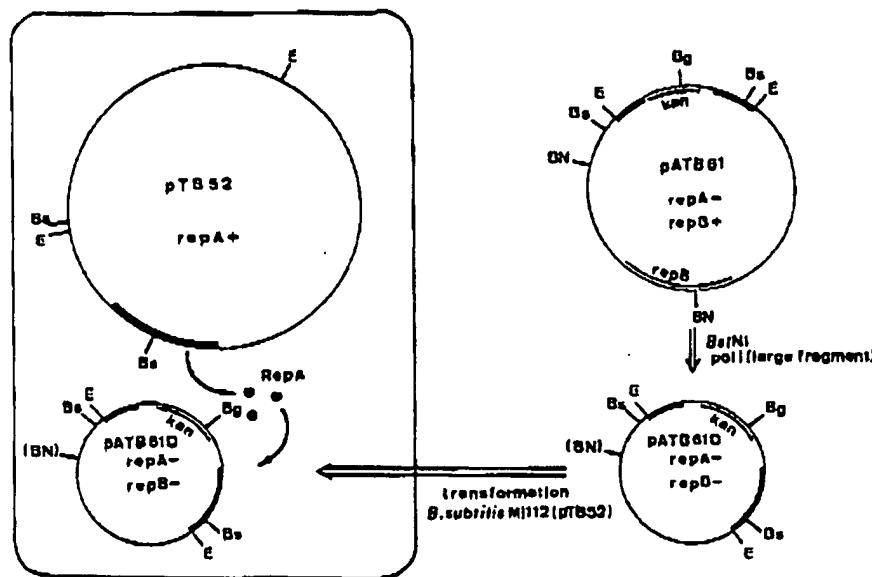


Fig. 5. *Cis-trans* test for the replication control of the *repA* plasmid by the RepA protein. BN, *Bsr*NI. For other symbols, see Fig. 3 legend

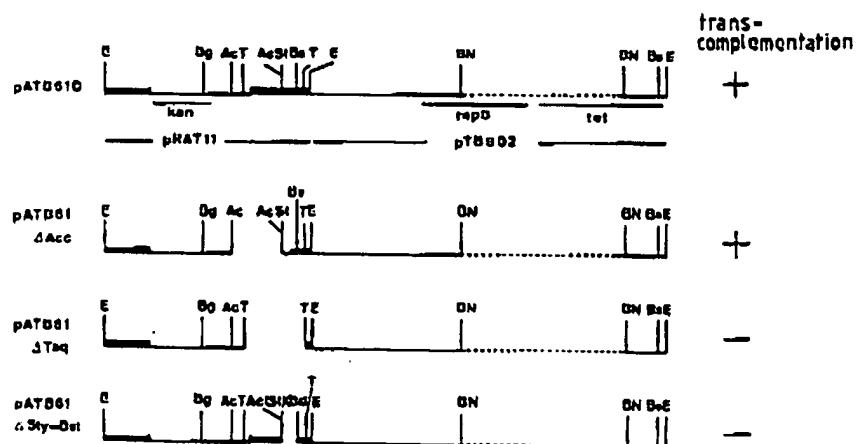


Fig. 6. Analysis of the RepA recognition region. Dotted lines indicate that the specific regions were deleted from the original *repB* plasmid in *Bacillus subtilis* by *Bsr*NI treatment to eliminate *repB* function. Ac, *Acc*; BN, *Bsr*NI; St, *Sty*. For other symbols, see Figs. 1 and 3

not the *repA* determinant. In fact, the copy number of pATB61 was as high as that of another *repB* plasmid, pTB902 (Imanaka et al. 1984). To eliminate the function of the *repB* replication determinant (Ano et al. 1986), the *Bsr*NI fragment was deleted from pATB61. The newly constructed plasmid DNA, named pATB61D, cannot replicate by itself.

pATB61D was used to transform recombination-deficient *B. subtilis* M1112 carrying pTB52 (*Tc'*, *repA* plasmid), and *Km'* *Tc'* transformants were obtained. When plasmid DNA was extracted from the transformants and analysed by agarose gel electrophoresis, both pTB52 and pATB61D were observed as discrete bands. Thus, the RepA protein is required for plasmid replication and functions in *trans*.

**Table 2.** Effect of coexisting plasmid on the activity of the penicillinase gene directed by the P<sub>A</sub> promoter

Plasmids	Penicillinase activity		Relative copy number of pPF301-PA
	Units/OD <sub>660</sub>	Ratio (%)	
pPF301-PA	72.3	100	1
pPF301-PA + pTB20	74.1	102	1.0
pPF301-PA + pTB902 (repB)	77.3	107	1.0
pPF301-PA + pTB52 (repA)	34.1	47	0.5

To localize the RepA binding site, deletion plasmids were constructed from pATB61 (Fig. 6), and used for a *cis-trans* complementation test as above. Plasmid, pATB61ΔAcc, lacking the *Acc* fragment (-252-447) was complemented, whereas those lacking the *Taq*I (-104-722) or *Sty*I-*Bsr*EII (452-612) fragments could not be complemented by pTB52. These results indicate that the RepA protein binds to the *Acc*-*Taq*I region (448-722) and the plasmid can replicate. This region contained the 3.5 direct repeats of 24 bp (Fig. 2); it was followed by another region, highly rich in A+T.

#### Expression of the repA gene

The *Hinf*I fragment (-228-218) containing the repA promoter was repaired with DNA polymerase I (large fragment), and inserted in the *Bam*HI site (after repairing the cohesive end by the polymerase) of the promoter-probe vector pPF301. *B. subtilis* MT113 carrying the recombinant plasmid pPF301-PA was transformed with pTB20, pTB902 (repB plasmid) or pTB52 (repA plasmid). The transformants were cultivated and penicillinase activity was assayed to assess the strength of transcription from the repA promoter (Table 2).

When pPF301-PA coexisted with either pTB20 or pTB902, penicillinase production was not influenced by these plasmids. Although *B. subtilis* carrying both pPF301-PA and pTB52 produced about 50% of that for the pPF301-PA carrier, the copy number of pPF301-PA was also reduced to about 50%. Therefore, it was inferred that the activity of the repA promoter is not affected by the repA plasmid.

#### Discussion

We determined the nucleotide sequence of the replication determinant (repA) of a low copy number *B. subtilis* plasmid. It was shown that the RepA protein (396 amino acids) is encoded on by the repA plasmid; it is required for plasmid replication and is a DNA binding protein.

The RepA binding site region (448-722) contains 3.5 direct repeats of 24 bp (Fig. 2), and is followed by a region which is considerably rich in A+T. Immediately upstream of the direct repeats, there is the specific sequence TTATC-CACA (536-544) that can be recognized by the DNA replication protein DnaA from *E. coli*, and that can serve as a replication origin region for plasmids R100, R1, CloDF13, P1 and F (Fuller et al. 1984). Consequently, the

sequence might also function as the replication origin region of the repA plasmid in *B. subtilis*, assuming that the sequence is recognized by the DNA replication protein from *B. subtilis* (Moriya et al. 1985). As a corollary, the fact that the repA plasmid can replicate only in *B. subtilis* and not in the thermophile *B. stearothermophilus* (Imanaka et al. 1984) could be supported.

When the repA promoter was cloned in the promoter-probe vector pPF301, *B. subtilis* carrying the plasmid pPF301-PA produced about 72 units of penicillinase per OD<sub>660</sub> unit. In contrast, when the P1 promoter of the repB plasmid was similarly cloned, 5,600 units of penicillinase per OD<sub>660</sub> unit was produced (Ano et al. 1986). The weak repA promoter activity would yield a low concentration of RepA protein, and this might have resulted in the low copy number of the repA plasmid.

When pPF301-PA coexisted with pTB52 (repA plasmid), the copy number of pPF301-PA decreased to about 50% (Table 2). The inserted *Hinf*I fragment (-228-218) contains a large inverted repeat (-103-30) which can potentially form a stem and loop structure. Since the inverted repeat region overlaps with the repA promoter sequence (-60-31), the region would have functioned as an incompatibility determinant. The probable existence of the incompatibility determinant could account for the reduction of copy number of plasmid pPF301-PA referred to above.

It has been demonstrated for many low copy number plasmids in *E. coli* such as R6K (McEachern et al. 1985), P1 (Chattoraj et al. 1985), F (Trawick and Kline 1985) and pSC101 (Vocke and Bastia 1985) that a plasmid-encoded protein can recognize direct repeats and regulate plasmid replication. Accordingly, a similar mechanism might be expected for the control of replication of the repA plasmid in *B. subtilis*.

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